J. Biochem. **137**, 477–485 (2005)
DOI: 10.1093/jb/mvi064

Characterization of Recombinant Amyloidogenic Chicken Cystatin Mutant I66Q Expressed in Yeast

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Received November 8, 2004; accepted January 31, 2005

Amyloidogenic chicken cystatin mutant I66Q (cC I66Q) was successfully secreted by yeasts *Pichia pastoris* and *Saccharomyces cerevisiae*. The soluble monomer and dimer forms of amyloidogenic cC I66Q were found in the culture medium, while large amounts of insoluble aggregate and polymeric form cC I66Q besides the monomer and dimer forms were secreted into the culture medium. The amyloidogenic cC I66Q showed a comparable circular dichroism spectrum to that of the wild cystatin, and the monomer form exhibited a similar level of inhibitory activity toward papain, but the dimmer form did not. During storage of amyloidogenic cC I66Q under physiological and acidic conditions, typical binding with Congo red and thioflavin T, and the formation of amyloid fibrils were observed, whereas the characteristic of similar amyloidosis was hardly detected for the wild recombinant cystatin.

Key words: amyloidogenic chicken cystatin, amyloid fibrils, *Pichia pastoris*, *Saccharomyces cerevisiae*, quality control.

Abbreviations: cC I66Q, chicken cystatin mutant I66Q; hCC, human cystatin; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase–polymerase chain reaction.

More than 20 proteins or their proteolytic products are now known to form amyloid fibrils in human proteins, and 7 of them are implicated in diseases of the central nervous system (*1*). Human cystatin C amyloid angiopathy (CAA) is a dominantly inherited disorder characterized by tissue deposition of amyloid in blood vessels that leads to recurrent hemorrhagic stroke (*2*). Postmortem examination of the brains of patients with hCC amyloid angiopathy shows severe deposition of amyloid within small arteries and arterioles of the leptomeninges, cerebral cortex, basal ganglia, brainstem, and cerebellum (*3*, *4*). Immunohistochemical studies of tissue sections from the central nervous system using polyclonal and monoclonal antibodies against human cystatin C have shown that the protein is deposited as amyloid in practically all arteries and arterioles (*5*, *6*). In addition, asymptomatic amyloid deposits can also be found in peripheral tissues, including skin, lymphoid tissues, salivary glands, and testes (*1*, *7*). The possibility that cystatin C may play a role in the pathogenesis of other amyloidosis was raised by the observation that it is present in both the cerebral amyloid lesions in FBD (familial British dementia) and the vascular amyloid lesions in AD (Alzheimer disease) (*8*).

Genetic mutation so as to substitute an amino acid is one of the different mechanisms likely able to influence the process of fibril formation. The L68Q variant of human cystatin C (hCC) is the causative agent of hCC amyloid angiopathy (*9*). Researches in last decade have verified that in pathological processes hCC variant L68Q can form highly stable and domain-swapped dimers at physiological protein concentrations, thus being responsible for part of the amyloidogenic deposits in the brain arteries of young adults, which leads to fatal cerebral hemorrhage (*10*). The disease also results in paralysis and development of dementia due to multiple strokes, and death from cerebral hemorrhage before 40 years of age (*11*).

Chicken cystatin has a number of similar characteristics to human cystatin C which makes them ideally suited for further study of cystatin C-type amyloidogenesis at the molecular level (*12*). However, there have been few papers on amyloidogenic chicken cystatin. Structurally, chicken cystatin and human cystatin C exhibit 62.5% similarity (*13*). Each cystatin consists of a five-stranded antiparallel β-sheet wrapped around a central helix, and both of them have two disulfide bonds and the insertion of an ~20 residue irregular structure between strands 3 and 4. Residue 66 in chicken cystatin, corresponding to residue 68 in human cystatin C, is located in the hydrophobic core of the protein. The amino acid substitution of isoleucine with glutamine at position 66 may cause altered intrinsic properties leading to amyloidosis. In addition, the higher thermodynamic stability of chicken cystatin must be of the experimental advantage over human cystatin C (*2*).

We have reported in a previous paper that the secreted amount of amyloidogenic lysozymes in *Saccharomyces cerevisiae* is extremely low compared with the wild type lysozyme because of their instability (*14*). However,

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cystatin was the first amyloidogenic protein to be shown to oligomerize in a stable form through a 3D domain swapping mechanism that is different from those for amyloidosis of lysozymes and other amyloidogenic proteins. Therefore, it seems likely that the secreted amount of amyloigenic cystatin may be higher than that of amyloidogenic lysozymes. Moreover, both *Pichia pastoris* and *S. cerevisiae* have subcellular organelles such as the endoplasmic reticulum and Golgi apparatus that allow for post-translational modifications such as folding, disulfide bridge formation and glycosylation. So far, no attempts to cause amyloidogenic chicken cystatin secretion in yeast have been reported. Therefore, it is very interesting to determine whether or not mature amyloid fibers can be formed by mutant cC I66Q secreted by yeast cells. Consequently, as a model of an amyloidogenic protein that is suitable for the study of amyloidosis in yeast and as a start to unravel the "mystery" of fibrillation of this unique amyloidogenic protein, we attempted to cause the amyloidogenic recombinant chicken cystatin secretion in yeast expression systems and investigated the physico-chemical properties of recombinant amyloidogenic chicken cystatin to clarify the mechanism of amyloidosis through this distinct amyloidogenic protein.

MATERIALS AND METHODS

Materials—Restriction endonuclease, T4 DNA ligase, Pyrobest DNA polymerase, a DNA sequencing kit and a mRNA selective PCR kit were purchased from Takara Shuzo (Japan). An RNeasy mini kit was purchased from Qiagen K.K., Japan. The pT7 Blue T-vector was from Novagen Merck (Germany). A QuickChange™ site-directed mutagenesis kit was purchased from Stratagene (USA). Synthetic oligonucleotides were purchased from Kurabo (Osaka, Japan). DNA sequencing was carried out with a Thermo Sequenase Core sequencing kit from Amersham Japan. An Easy Select Pichia expression kit was purchased from Invitrogen Corp. (USA). CM-Toyopearl resin was a product of Toso (Tokyo, Japan). Sephadex G-75 Superfine resin was purchased from Pharmacia (Sweden). All other chemicals were of analytical grade for biochemical use.

Bacterial Strains and Plasmids—*E. coli* XL-1 blue [*lac* (F′ proAB, lacI^{q}ZΔM15, Tn10 (Tet^R)) *rec*A1, *end*A1, *gyr*A96, *thi*-1, hsdR17, *sup*E44, *rel*A1, and Top10F′ (F′[*pro*AB, *lac*I^q, *lac*ZΔM15, Tn10(Tet^R)] *mcr*A, Δ(*mrrhsd*RMS-*mcr*BC), 80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, λ*ara*D139, Δ(*ara-leu*) 7697, *gal*U, *gal*K, *rps*L (Str^R), *end*A1, *nup*G) supplied by Amersham Japan were used as host cells in the cloning experiments. *S. cerevisiae* haploid strain F1105 (MATα*ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100*) was provided by Amy Chang, Albert Einstein College of Medicine, USA. *P. pastoris* X-33 (wild type, *mut*+) and the pPICZαA expression plasmid vector were purchased from Invitrogen. pYG100, an *E. coli*–yeast shuttle vector, was provided by Dr. K. Matsubara of Osaka University.

Construction of Cystatin Expression Vectors—Total RNA was isolated from chicken oviduct using an RNeasy mini kit. A DNA fragment encoding chicken cystatin was prepared from total mRNA by two step reverse transcriptase RT-PCR using mRNA-selective PCR kit. For insertion of the cystatin cDNA into the *P. pastoris* expression vector, the cystatin cDNA containing *Xho*I and *Xba*I sites was first cloned into subcloning vector pT7 blue T-vector, and then the cystatin cDNA was ligated with pPICZαA, an expression vector containing an *AOX1* promoter that allows methanol-inducible and high-level expression in yeasts. Finally, the *Sac*I linearized DNA was transformed in *P. pastoris* cells by electroporation. For insertion of *S. cerevisiae* expression vector pYG100, the cystatin cDNA fragments incorporating the *S. cerevisiae* α-factor signal with a *Sal*I site at both ends were cloned into subcloning vector pT7 blue T-vector, and then the cystatin cDNA was ligated in the *Sal*I site of pYG100.

Quick Change Site-Directed Mutagenesi—Quick change site-directed mutagenesis of cystatin cDNAs was carried out by PCR methods as follows. Template DNA in subcloning vector pT7 blue T-vector (50 μg), Pyrobest DNA Polymerase (0.5 μl:5U/μl), Pyrobest reaction buffer (5 μl), and dNTP mixture (1 μl:2.5 mM) were used for PCR. *Dpn*I restriction enzyme (1 μl:10U/μl) was used for the digestion of template DNA. The synthetic oligonucleotide primers for mutant I66Q were 5′-CTG CAG GTT GAG CAG GGT CGG ACA ACT T-3′ (sense) and 5′-AAG TTG TCC GAC CCT GCT CAA CCT GCA G-3′ (antisense) as sense and antisense primers, respectively. PCR was performed with three-temperature cycling (98°C for 10 s, 66°C for 1 min and 72°C for 8 min) for 18 cycles. *E. coli* XL-1 blue competent cells were used for the ligation of PCR products. After PCR, the methylated parental DNA template was digested with *Dpn*I for 2 h at 37°C. and then the reaction mixture containing DNA with the designed mutation was transformed into *E. coli* XL-1 blue cells to repair the nicked DNA. Colonies were selected on an LB agar plate containing 50 μg/ml of Carbenicillin. The mutations were confirmed by DNA sequencing.

Expression of Recombinant Chicken Cystatin—After the pPICZαA/cC plasmid had been transformed into the *P. pastoris* X-33 expression host, the expression vector was integrated into the genomic DNA due to the existence of the AOX promoter sequence. The successfully transformed colonies were cultivated for 18 h in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Subsequently, the cells were harvested by centrifugation (1,500 × *g*, 5 min) and the cell pellets were suspended in 200 ml YPM medium (1% yeast extract, 2% peptone, and 0.5% methanol). To induce expression of the recombinant chicken egg white cystatin, these cultures were incubated for 3 days at 30°C with shaking, 100% methanol being added to a final concentration of 0.5% methanol every 24 h. On the other hand, the expression vectors pYG100/cC were introduced into *S. cerevisiae* F1105 by the LiAc/Single Strand carrier DNA/PEG method (*15*). The over-expressed proteins exhibiting the highest activity levels were screened and propagated from single colonies inoculated into 3 ml of yeast minimal medium and incubated for 3 days at 30°C with shaking. Each preculture was subcultured into 600 ml of the same medium in a flask (1,000 ml) and incubated for another 3 days at 30°C with shaking.

Purification of Recombinant Chicken Egg White Cystatin—The cultivation media were first centrifuged at 3,000 × *g* for 10 min to remove the cells. The supernatant was centrifuged at 11,900 × *g* for 40 min to collect the

insoluble protein aggregates, and then the supernatant was fractionated with various ammonium sulfate concentrations to separate the monomer, dimer and polymer forms of cystatin. The proteins precipitated on the addition of various concentrations of ammonium sulfate were collected by centrifugation at 11,900 × *g* for 40 min, and the precipitate was dissolved in 5 ml of phosphate buffer (50 mM, pH 7.0). The resultant solution was desalted by dialysis against phosphate buffer (50 mM, pH 7.0) overnight. The samples dissolved from the precipitation step were diluted and applied on a CM-Toyopearl column. The absorbed recombinant cCs were eluted with a gradient manner using 0–0.5 M sodium chloride in 20 mM Tris-HCl buffer, pH 7.5. The protein content of each fraction was determined by measuring the absorbance at 280 nm. The fraction containing the protein was collected and dialyzed against deionized water to remove salt at 4°C. In order to further purify the dimeric and monomer forms of recombinant cCs, the solution was applied to a Sephadex G-75 superfine column (3 cm × 100 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) comprising 0.2 M sodium chloride, 0.01% BRIJ·35 and 0.02% sodium azide. Finally, the protein content of each fraction was determined by measuring the absorbance at 280 nm. After precipitation of samples with different concentrations of ammonium sulfate, the precipitates were dissolved and subjected to SDS-PAGE analysis. In order to determine the protein levels, the intensities of the Coomassie Blue stained bands were determined by densitometric measurement using native egg white cystatin (Sigma) as the protein standard. The concentration of native chicken egg white cystatin was determined by the Lowry method (*16*). The secreted amount of the monomer was calculated based on the standard protein band intensity, and the secreted amount of oligomers was calculated.

SDS-PAGE and Native PAGE—SDS-PAGE was conducted according to the method of Laemmli (*17*) using a 15% acrylamide separating gel and a 5% stacking gel containing 1% SDS. Samples were heated at 100°C for 5 min in Tris-glycine buffer (pH 6.8) containing 1% SDS and 1% 2-mercaptoethanol. Electrophoresis was carried out at a constant current of 10 mA for 5 h using an electrophoretic Tris-glycine buffer containing 0.1% SDS. Native-PAGE was performed according to the method of Davis (*18*). Samples for native-PAGE were prepared in sample buffer without SDS and without 2-mercaptoethanol. Samples were subjected directly without further treatment to non-denaturing polyacrylamide gel electrophoresis [5% (w/v) stacking and 7% resolving gel] at a constant current of 5 mA for 4 h at room temperature. SDS-PAGE and Native-PAGE gel sheets were both stained with Coomassie Brilliant Blue R-250.

Western Blotting—After 15% SDS polyacrylamide gel electrophoresis, protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane for 1 h at 100 mA in blotting buffer (20 mM glycine, 25 mM Tris, 20% methanol). Subsequently, non-specific binding sites were blocked by immersing the PVDF membrane in 0.05% Tween 20 in phosphate buffer (PBS) containing 1% BSA for 1 h at room temperature on an orbital shaker followed by exposure to rabbit anti–chicken cystatin antiserum (1×10^{-4} in 0.05% Tween 20) in Tween-PBS for 1 h at room temperature. The membrane was further exposed to anti–rabbit peroxidase conjugate (1×10^{-4} in Tween-PBS) for 1 h at room temperature. Immunodetection of recombinant cystatins was carried out with an ECL-Plus kit (Amersham Pharmacia Biotech, UK). The chemiluminescence intensities associated with the various protein bands were determined by exposing the filter to X-ray film with a Hypercassette.

TOF-MS—TOF-MS analysis was performed with a Voyager DE/PROJ (Perseptive Biosystems, USA). The matrix, 3,5-dimethyl-4-hydroxciammic acid (sinapic acid), was dissolved in a reaction solution comprising equal volumes of acetonitrile and 0.1% TFA for recombinant chicken cystatins. The sample concentration was 1.5 mg/ml for I66Q mutant polymers.

Circular Dichroism (CD) Analysis—Far-ultraviolet (200–260 nm) circular dichroism (far-UV CD) spectra were measured to estimate the conformational change in recombinant cystatins according to the method of Kato and Takagi (*19*). Recombinant cystatin solutions were adjusted to 0.01 mg/ml with distilled water. CD spectra were recorded at 25°C on a J-600 spectropolarimeter (Jasco., Tokyo, Japan) with a 1.0 cm cuvette. The percentages of the different secondary structure motifs were calculated from the CD data by means of the CD Estima algorithm (*20*). The conformational stability of recombinant cystatins was determined from denaturation curves, which were obtained from the change in the ellipticity of CD spectra at 222 nm (the characteristic wavelength of the ordered structure of the α-helix) during heating in the range of 60°C to 95°C at the rate of 1°C/min. The melting temperature (T_m) was determined from the transition point of the denaturation curve.

Inhibitory Activity—The methods used for active site titration and determination of equilibrium constants for the dissociation (K_i) of complexes between cystatin and papain have previously been described in detail (*21*). The cysteine protease inhibitory assay was performed using papain as the target enzyme and Z-Arg-Arg-NNap as its substrate with some modifications, the active concentrations of the wild type and mutant cC I66Q were determined by titration with papain. The papain was previously active site-titrated using L-3-carboxy-2,3-*trans*-epoxypropionylleucylamido(4-guanidino)-butane (E64). The assay buffer was 73 mM sodium phosphate, pH 6.0, containing 2 mM cysteine and 1 mM EDTA to activate the enzyme. The absorbance of the cleaved 2-naphthylamine was measured at 520 nm with a Hitachi U-2000 spectrophotometer.

The inhibitory activity toward papain was also determined by substrate SDS-PAGE (*22*). 15% polyacrylamide gels containing 0.1% w/v casein were used for the activity assay. 0.5 μg of sample protein was applied to each well in the gels. After electrophoresis, the gels were prewashed with 2.5% Triton X-100 at least two times for 30 min to remove SDS, and then incubated in 0.10 M phosphate buffer containing 2 mM cysteine, 1 mM EDTA, pH 6.0, and papain (0.01 mg/ml) at 40°C for 150 min. Cystatin activity was abolished with the staining solution, 0.01% Coomassie Brilliant Blue, 40% methanol, and 10% acetic acid. Active cystatin zones were visualized as intense blue bands against a clear background on the gels after destaining with 25% ethanol and 10% acetic acid.

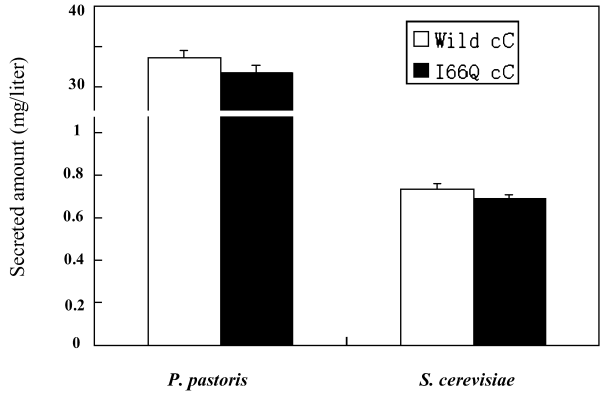

Fig. 1. **Secreted amounts of the wild type and amyloidogenic mutant I66Q in both *P. pastoris* and *S. cerevisiae*.** Wild type recombinant cC, white columns; Amyloidogenic I66Q cC, black columns. The vertical bars indicate the standard deviation (n = 3).

Congo Red Assay—Congo Red binding to amyloid aggregates was examined as described previously by Klunk *et al.* (*23*, *24*). Aliquots of 10 mg/ml of prepared protein samples (10 μl) were diluted in the reaction buffer (5 mM sodium phosphate/150 mM NaCl, pH 7.4), which contained 5 μM Congo red (final reaction volume, 1 ml). The Congo red solution was freshly prepared and filtered through a 0.2 μm filter before use. The reaction samples were thoroughly mixed and incubated at room temperature for at least 30 min before recording the absorbance spectra. The absorbance at 480 and 540 nm was determined, and the level of Congo Red binding was determined using the equation:

$$\text{CRB}\ (\mu\text{M}) = (A^{540}/25{,}295) - (A^{480}/46{,}306)$$

Thioflavin T Fluorescence Measurements—Thioflavin T fluorescence has been used to trace the kinetics of fibrillation (*25*). Wild or I66Q cystatin solutions (1 μg/μl) in 66 mM glycine–hydrochloric acid buffer, pH 2.0, containing 5% EtOH and 0.04% NaN_3 were incubated for 35 days. Samples of 20 μl were mixed with 80 μl of 2.5 mM thioflavin T solution in 10 mM potassium phosphate containing 150 mM NaCl, pH 7.0. Fluorescence intensities were measured at an excitation wavelength of 440 nm and an emission wavelength of 510 nm with a fluorescence spectrophotometer (Wallac 1420 ARVOsx, Perkin Elmer).

Transmission Electron Microscopy—Samples of wild type or I66Q variant chicken cystatin (1.0 μg/μl) were incubated for 35 days at 57°C in 100 μl of 66 mM glycine–hydrochloric acid buffer, pH 2.0, containing 5% EtOH and 0.04% NaN_3. After incubation, the prepared samples were adsorbed on carbon-coated copper grids and negative stained with 2% phosphate tangustanic acid, pH 6.0. Micrographs were recorded at a nominal magnification of 1,000 to 20,000 with electron microscope (Hitachi, H-7600) operating at 80 kV.

RESULTS

Amyloidogenic Mutant Cystatin I66Q and Wild Type Cystatin Secreted in Similar Amounts by Both S. cerevisiae and P. pastoris—The secretion of the recombinant cC I66Q and wild type cystatins was examined in both *S. cerevisiae* and *P. pastoris* after 3 days cultivation when the secreted amounts were the maximum. The total secreted amount of the I66Q mutant was similar to that of the wild type cystatin secreted with both *P. pastoris* and *S. cerevisiae* expression systems (Fig. 1). This suggests that the amyloidogenic cystatin might be secreted in a relative stable form. As shown in Fig. 1, the secreted amounts of both the wild type and amyloidogenic cystatins in *S. cerevisiae* are much lower than those secreted in the *P. pastoris* expression system. Therefore, in the following experiments, *P. pastoris* was chosen for large scale expression of both the wild type and amyloidogenic mutant cystatins.

Electrophoretic Patterns of Soluble and Insoluble Forms of Amyloidogenic Mutant Cystatin I66Q Secreted by P. pastoris—The transformed yeasts carrying both the wild type and I66Q mutant cDNAs were cultivated at 30°C for 3 days. After 3 days, the supernatant of the cultivation medium was directly subjected to native PAGE and SDS-PAGE (Fig. 2). After cultivation of the transformant of mutant cC I66Q, the large amount of insoluble aggregate in the culture medium was collected by centrifugation at 11,900 × g for 30 min, while such an insoluble aggregate was not found in the culture medium of the

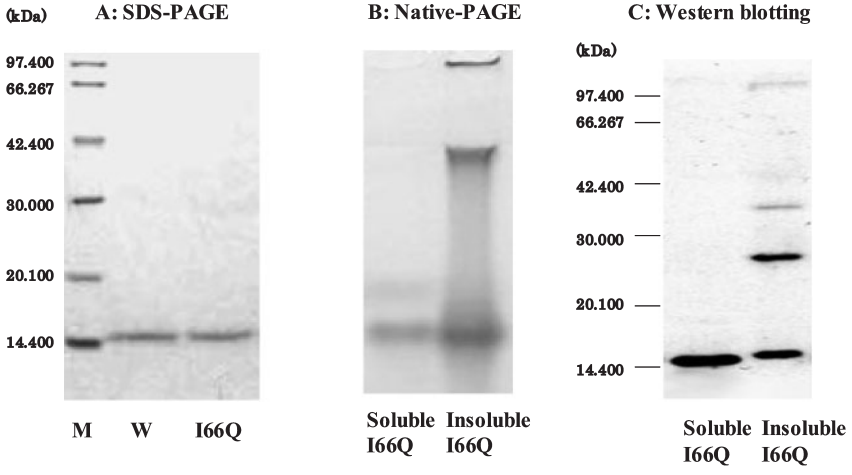

Fig. 2. **Amyloidogenic mutant I66Q cC and wild type cC secreted as different forms by *Pichia pastoris* X-33.** (A) SDS-PAGE analysis of cultured medium of the wild and amyloidogenic mutant I66Q cC. M, molecular weight markers; W, wild recombinant cC; I66Q, recombinant cC mutant I66Q. (B) Native-PAGE analysis of amyloidogenic mutant I66Q soluble and insoluble samples; both the native and SDS-PAGE gels were stained with Coomassie Brilliant Blue. (C) Western blotting with anti-cystatin polyclonal antibodies after reducing SDS-PAGE. The samples of soluble and insoluble I66Q corresponded to those on native PAGE, respectively. The arrows indicate the molecular weight markers in panel A.

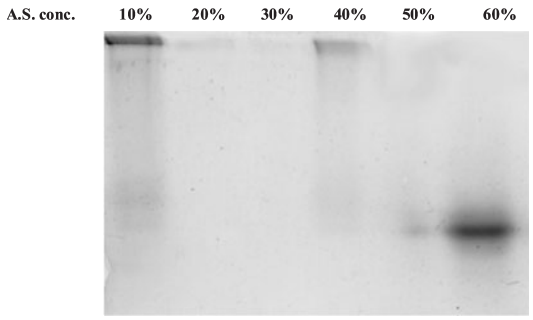

Fig. 3. **Native-PAGE analysis of amyloidogenic chicken cystatin secreted by *Pichia pastoris* after precipitation with various concentrations of ammonium sulfate (A.S.).** The gel sheet was stained with Coomassie Brilliant Blue. 10, 20, 30, 40, 50, and 60% are ammonium sulfate concentrations.

transformant of wild type cystatin. The native PAGE pattern showed the presence of polymeric forms in the insoluble aggregate in that of mutant cC I66Q (Fig. 2B). Western blotting after SDS-PAGE showed that the insoluble aggregate mainly consisted of the monomer and dimer forms (Fig. 2C). These electrophoresis results suggest that after SDS treatment and boiling, most of the polymer and insoluble aggregate of amyloidogenic I66Q were converted to the monomeric form (the band at approximately 14 kDa) or dimeric form (the band at approximately 28 kDa). It is noticeable that considerable amounts of dimer and oligomer still remained. The molecular weights of the dimer and monomer of I66Q were confirmed by TOF-MS analysis, being calculated to be 13,283 Da and 26,397 Da, respectively, which are comparable to those of the native cystatin.

The Secreted Amounts of the Insoluble Aggregate and Polymeric Forms of Amyloidogenic Mutant Cystatin I66Q Increase during Culturing—To further elucidate the mode of assembly of amyloidgenic mutant cC I66Q, native PAGE analysis of the soluble culture medium was performed for the fraction obtained on salting-out with ammonium sulfate. Ammonium sulfate precipitation was carried out right after cultivation on a given day. The protein fraction precipitating between 10 to 40% saturation comprised mainly the polymeric form, while the fraction precipitating between 50 to 60% saturation comprised the monomeric form (Fig. 3). On the basis of the results in Fig. 3, the fractions precipitated with 10–40, 40–50, and 60% saturation were collected and then subjected to SDS-PAGE to determine the content of each fraction (Fig. 4, A, B, and C). The protein bands were confirmed again by Western blotting with anti-cystatin polyclonal antibodies (Fig. 4D). As shown in Fig. 4E, the amount of the monomer (white columns) remained constant during cultivation, while both the polymeric form (dark or light gray columns) and insoluble aggregate (black columns) increased with the cultivation time.

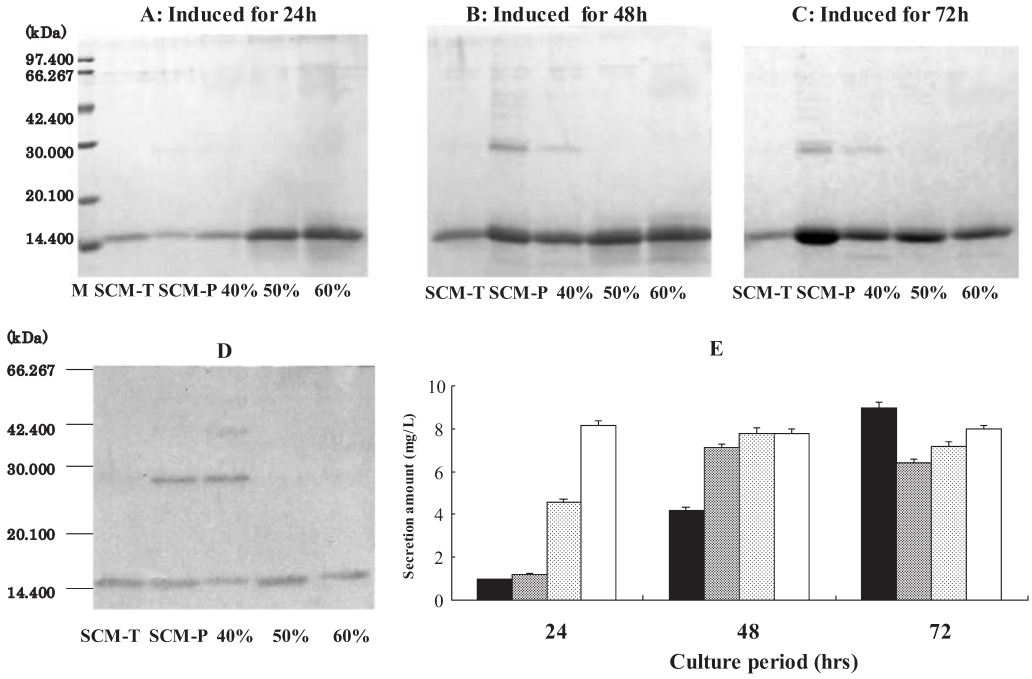

Fig. 4. **SDS-PAGE analysis of changes in the secreted amounts of different forms of I66Q cC in *P. pastoris* induced for 24 h (A), 48 h (B), and 72 h (C).** After collection of the insoluble precipitate, the left supernatant was subjected to fractionation by ammonium sulfate precipitation at 40% saturation, followed by 50% and 60% saturation in the second step to precipitate different forms of I66Q cC. M, molecular weight markers; SCM-T, total proteins in supernatant of culture medium; SCM-P, precipitate of the culture medium supernatant (after removing cells, the supernatant was centrifuged at 11,900 × *g* for 40 min to collect the insoluble protein aggregate). The gel sheets were stained with Coomassie Brilliant Blue. (D) Western blotting with anti-cystatin polyclonal antibodies after reducing SDS-PAGE. The samples correspond to those in panel A, respectively. (E) Quantitative determination of the changes in the secreted amounts of different forms of I66Q cC in *P. pastoris* induced for 24 h (A), 48 h (B), and 72 h (C). Black columns, insoluble aggregate; dark grey columns, 40% AS precipitated proteins; grey columns, 50% AS precipitated proteins; white columns, monomer. The vertical bars indicate the standard deviation (n = 3).

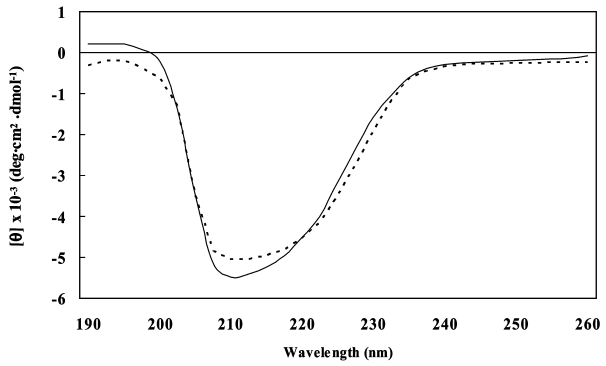

Fig. 5. **Far-ultraviolet–CD spectra of monomeric forms of the wild and I66Q mutant cystatins.** The spectra were recorded at 25°C and pH 7.0 on a J-600 spectropolarimeter. Wild recombinant cC, broken line; amyloidogenic mutant I66Q recombinant cC, solid line.

Amyloidogenic Mutant Cystatin I66Q Shows a Slightly Different Secondary Structure from That of the Wild Type Cystatin—The Far-UV CD spectrum of amyloidogenic I66Q cC is comparable to that of the wild type cystatin (Fig. 5). A broad featureless minimum at 218 nm strongly suggests a great contribution of β-structure to the spectrum. Although the CD spectrum of I66Q cC closely coincided with that of wild type cC, the absolute minimum for amyloidogenic mutant I66Q shows a slight blue shift as compared with wild cC, indicating a certain level of change in β-structure. The contents of α-helix and β-sheet of wild type cC were calculated to be 21 and 42%. The mutant cC I66Q had a lower content of α-helix (14%) and a higher content of β-sheet (45%) compared to the wild cC. These findings strongly indicate that the amyloidogenic mutant cC I66Q has a slightly more unfolded structure than that of the wild type protein.

Table 1. **Equilibrium constants for dissociation (K_i) of the complexes between papain and the different recombinant cystatins.**

	$K_i \pm$ SD (pM)	n
Native cC	2.0 ± 0.058	5
Wild type cC	2.5 ± 0.066	5
Wild type cC monomer	2.7 ± 0.032	5
I66Q cC dimer	>20 μM	6

The standard deviation (SD) and number of measurements (n) used to calculate the mean K_i values given are indicated.

The Amyloidogenic Mutant Cystatin I66Q Dimer Exhibits a Loss of Inhibitory Activity toward Papain Compared with the Wild Type Cystatin—To determine and compare the functional activities of the wild type and I66Q mutant cystatins, the purified proteins were subjected to inhibitory activity analysis as to papain. The equilibrium dissociation constants (K_i) of the complexes with papain were determined by inhibition under steady-state conditions (Table 1). Both the wild type and I66Q mutant recombinant cystatin monomers showed efficient inhibitory activity toward papain, K_i being 2.5×10^{-12} M and 2.7×10^{-12} M, respectively. Whereas in the case of the I66Q cC dimer, no significant inhibition was observed compared with the two kinds of cC monomers, indicating a very weak interaction between the cC I66Q dimer and papain.

The inhibitory activity toward papain was also detected on substrate SDS-PAGE and substrate native-PAGE assay. The wild type cC monomer, cC I66Q monomer, as well as the polymer sample of cC I66Q were

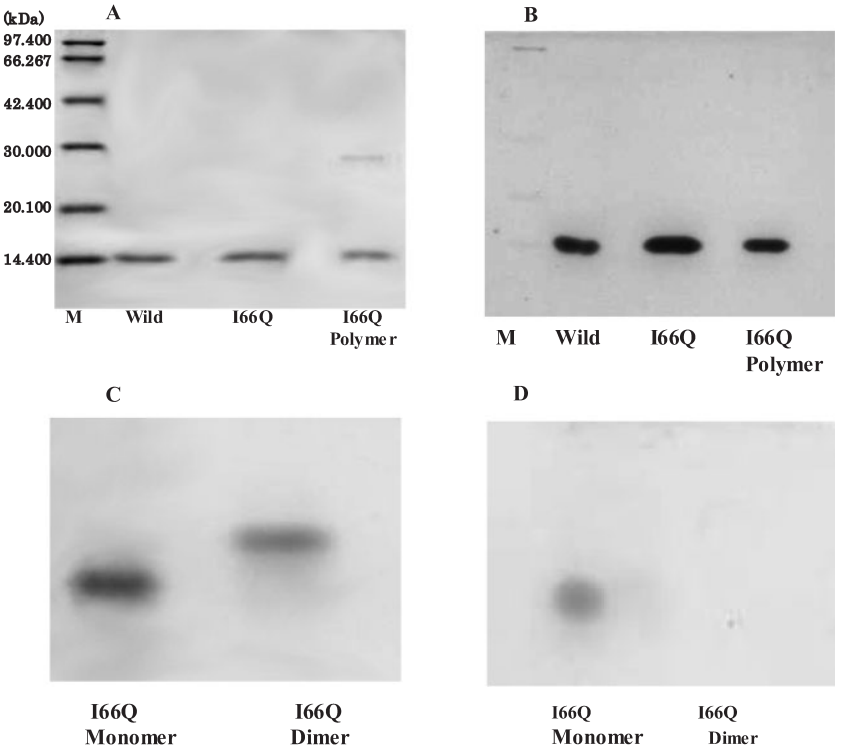

Fig. 6. **Substrate SDS-PAGE and native PAGE of the wild and I66Q mutant cystatins.** A, SDS-PAGE; B, substrate SDS-PAGE; C, native-PAGE; D, substrate native-PAGE. M, molecular weight markers; Wild, wild recombinant cC; I66Q, recombinant cC mutant I66Q. A 15% polyacrylamide gel containing 0.1% w/v casein was used for the substrate PAGE. 0.5 μg of a sample protein was applied to each well of the gels. The bands of unhydrolyzed casein (Coomassie Brilliant Blue–stained) indicate the existence of cystatin. Both native-PAGE and SDS-PAGE gel sheets were stained with Coomassie Brilliant Blue.

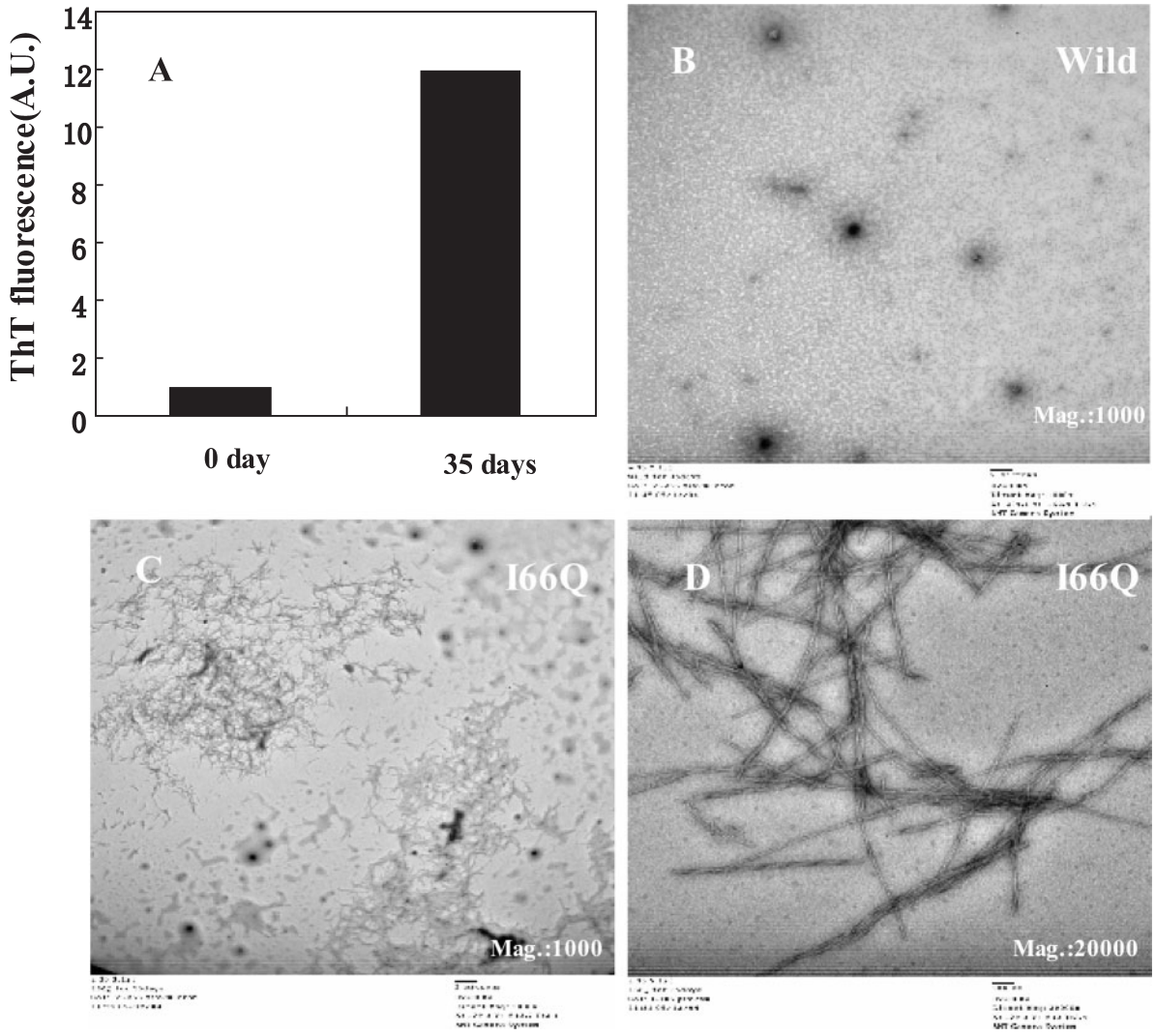

Fig. 7. **(A) Relative thioflavin T fluorescence values after 35 days incubation of I66Q cC sample (1 μg/μl) at 57°C in 66 mM glycine-hydrochloric acid buffer, pH 2.0, containing 5% EtOH. (B, C, and D) Electron microscopy of the wild (B), and I66Q (C and D) recombinant chicken cystatins.** Samples were incubated for 35 days at 57°C, in 66 mM glycine–hydrochloric acid, pH 2.0, containing 5% EtOH. After incubation, suspensions of the wild and I66Q recombinant cystatins were negatively stained and then examined by electron microscopy.

applied, and the polymer sample reverted to the monomer and dimer after the SDS treatment, as shown in Fig. 6, A and B. While the monomer forms of the recombinant wild and I66Q cCs remained active, the dimer of amyloidogenic mutant I66Q cC exhibited little remaining inhibitory activity toward papain (Fig. 6C). Similar result was obtained on substrate native PAGE developed from the original substrate SDS-PAGE method (Fig. 6D).

Amyloidogenic Mutant Cystatin I66Q Shows Fibrillogenicity In Vitro Whereas the Wild Type Cystatin Can Not Form Fibers under the Same Conditions—The fibrillogenicity of the wild type and cC I66Q was studied by thioflavin T fluorescence and negative staining electron microscopy analysis. Several conditions for fibril formation were investigated, including different buffer pHs (2.0 to 7.0), different temperatures (37°C, 57°C), and ethanol addition or non addition. Fibrils of wild type cystatin C were found to be produced in solutions of low pH, and the production was accelerated by high temperature (*1*, *26*) and ethanol addition (*27*). After 35 days incubation of a I66Q cC sample at 57°C in 100 mM hydrochloric acid–potassium chloride buffer, pH 2.0, containing 5% EtOH , the relative thioflavin T fluorescence value was much higher than the value before incubation (Fig. 7A). Electron microscopy analysis also showed that cC I66Q clearly formed typical amyloid fibrils on prolonged incubation (Fig. 7, C and D), while wild type cC failed to form fibrils (Fig. 7B). The width of the detected fibroid material was calculated to be in the range of 70–90 Å, which closely matches the widths of fibers observed for many amyloidogenic proteins, including those of human cystatins (*12*).

A distinctive property of amyloid fibers is their capacity to bind the dye Congo Red. The spectral shift accompanied by Congo red binding to amyloid proteins was used to monitor the time course of cystain assembly during incubation of the soluble form of the protein under physiological conditions (37°C, pH 7.4). The rates of

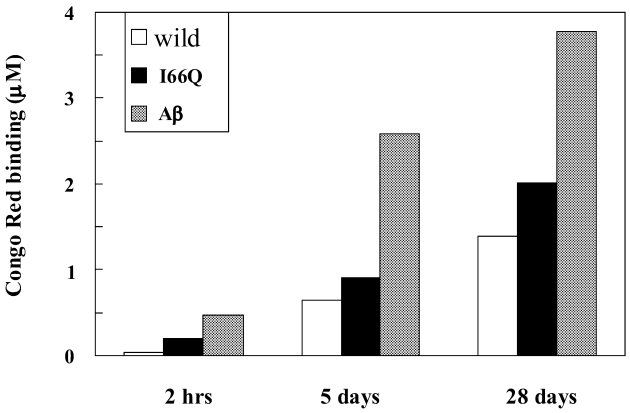

Fig. 8. **Time course of Congo Red binding of wild cC (white columns) and I66Q cC (black columns).** Aβ peptide (grey columns) was used as a positive control. Congo Red binding was calculated as described under "EXPERIMENTAL PROCEDURES" and is represented as the means of duplicate determinations.

Congo red binding of both the wild type and I66Q recombinant cCs increase with the incubation time, whereas cC I66Q shows a higher Congo red binding capacity than that of the wild type cC (Fig. 8). Analysis of the Congo red binding time course showed that 2.0 ± 0.05 μM/liter Congo red bound to the recombinant cC after 28 days incubation at 37°C, pH 7.4, which is higher than the value for wild type cC but lower than that for Aβ42.

Overall, these results suggest that amyloidogenic mutant cC I66Q shows a high propensity to form fibers not only under harsh conditions but also under physiological conditions.

DISCUSSION

A number of amyloidogenic proteins are unstable under physiological conditions. Our previous paper showed that the secretion of amyloidogenic lysozyme by *S. cerevisiae* is extremely low compared with that of wild type lysozyme due to the unstable conformation (*14*). In contrast, amyloidogenic mutant cC was secreted at a similar level to that of wild type cC. Considering our CD spectrum results, amino acid replacement does not lead to a significant secondary structural change. This intriguing finding possibly indicates that molecular chaperones in the ER of yeast may not distinguish the slightly structure change between amyloidogenic and wild type recombinant cystatins. Therefore the quality control system in the cell may not work, thus leading to a similar secreted amount of amyloidogenic variant cC to that of wild type cC.

It has been reported that the L68Q variant of human cystatin C dimerizes under physiological conditions through 3D domain-swapping (*4*). Similarly, the dimeric form of I66Q cystatin may be formed immediately after secretion. Moreover, the polymer can be further assembled from domain-swapped dimers via the interface between strands 1 and 5. Thus, large amounts of insoluble and soluble polymeric forms of recombinant I66Q cC can be detected after expression. The time course of the secreted amounts of different forms of I66Q cC in the culture medium demonstrated that the dimer and polymeric forms of I66Q cC gradually accumulated to form the insoluble aggregate of amyloidogenic cystatin as the culture time increases. On the other hand, the oligomeric form and insoluble aggregate are not formed for the wild cystatin.

When nonpolar hydrophobic isoleucine is replaced by polar hydrophilic amino acid glutamine, the original hydrophobic core may be distorted, thus causing dimerization of the original cystatin fold, although this dimerization involves no structural rearrangement of the main fold. Changes are confined to the region containing the protease inhibitory active site, *i.e.*, the loop between strands 2 and 3, while the original wedge-shaped papain inhibitory domain is formed by the N-terminal segments and two loop-forming segments. Therefore, the absence of loop 1 and disruption of the original cystatin fold may lead to the lack of inhibitory activity of the amyloidogenic cystatin dimer.

Although there are no big differences in the secondary structure and secreted amounts of wild and I66Q cCs in yeast, the fibrillogenicity of the wild type and I66Q cC *in vitro* are fairly different. Immature protofibrils as well as mature fibrils can be detected on negative staining electron microscopy after over one month inbubation, however, no such fibroid material is observed for wild cC. Meanwhile, the percentages of the different secondary structure motifs calculated from the CD data and the time course of Congo red binding revealed an increase in the β-structure of I66Q cC. This structural change is caused by the exposure of more hydrophobic surface in the aggregates, which renders them more prone to further aggregation. It has been reported that a number of amyloidogenic proteins, such as α-synuclein and the yeast prion Sup35, may require the formation of an initial nucleus made up of a number of unfolded molecules before they can be rearranged into the folded β-structures that make up amyloid fibers (*28*). Recently, Vattemi *et al.* reported that human cystatin C was colocalized with Aβ on 6–10 nm amyloid-like fibrils and floccular material in AβPP-overexpressing cultured normal human muscle fibers (*29*). In addition, Sanchez *et al.* also reported that analysis of the cystatin C level in cerebrospinal fluid could be useful as a pre-mortem indicator for the diagnosis of Creutzfeldt-Jakob disease (*30*). Therefore, it is conceivable that fibrillation of recombinant I66Q cC may also need some kind of initial nucleus.

Our experiment demonstrated that mutant I66Q cC but not the wild type cC can form mature amyloid fibers *in vitro*, however, it maintains a relatively stable conformation *in vivo*, indicating that *in vivo* protein amyloidogensis through 3D domain-swapping is a distinct mechanism that is fairly different from other amyloidogenesis mechanisms.

This work was supported by a Grant-in-Aid (14037244) for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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